Journal of Medical Virology 59:536–540 (1999)

Semiautomated Typing of Human Papillomaviruses by Restriction Fragment Length Polymorphism Analysis of Fluorescence-Labeled PCR Fragments

Tian-Shiang Wang,[1] Huar-Fen Chou,[1] Wei-Ming Liu,[2] and Kong-Bung Choo[1]*

[1]*Center for Molecular Medicine, Department of Medical Research and Education, Veterans General Hospital-Taipei, Taipei, Taiwan*

[2]*Department of Obstetrics and Gynecology, Veterans General Hospital-Taipei, Taipei, Taiwan*

A simplified version of a PCR-based reductional restriction fragment length polymorphism (rRFLP) approach for typing of human papillomaviruses (HPVs) is described previously [Wang et al., 1997]. It is achieved by the use of a biotin-labeled primer in PCR which, on restriction digestion and staining, is associated with only a single restriction fragment. In this report, we describe a further development of the rRFLP approach with the use of a fluorescence-labeled primer in PCR and fragment detection by laser scanning in an automatic sequencer. HPV typing is achieved by computer-assisted matching of the fluorescence-labeled rRFLP patterns with a database of rRFLP patterns of all known anogenital HPV types. On analysis of the typing of 133 HPV-positive cases using this procedure, 20 different HPV types were detected in exfoliated cervical cells in PAP smear samples derived from Taiwanese women. The results indicate the existence of a heterogeneous population of HPV types in Taiwan. Although most cases were associated with the more common HPV types, a significant fraction (about 20%) of the HPV types detected was related to the less common genotypes, which are often not included in commercial kits available for HPV typing. The results indicate the importance of covering as many HPV types as possible in clinical HPV genotyping protocols. ***J. Med. Virol. 59:536–540, 1999.***

KEY WORDS: human papillomavirus; typing; RFLP; fluorescence labeling

INTRODUCTION

Anogential human papillomaviruses (HPVs) are found to be closely associated with many forms of dysplasias and neoplasias of the anogenital tract [de Villiers, 1989; zur Hausen, 1994]. Over 90% of cervical cancers are associated with this virus. To date, over 40 distinct HPV types have been described that form the mucosal anogenital group of HPVs, in contrast to the cutaneous HPV group. Physically, different HPV types are distinguished by >10% differences in nucleotide sequence homology. Furthermore, due to differences in oncogenic potentials, HPVs are also categorized as low- or high-risk subgroups with different clinical outcomes [Lorincz et al., 1992; Jacobs et al., 1995]. Thus, while it is clinically important to detect the presence of HPV infection in a sample, accurate HPV typing is also essential. HPV detection is thus an additional diagnostic marker for the detection of high-risk patients for the development of cervical lesions, in particular cervical cancer.

A number of methods based on molecular hybridization using HPV type-specific probes or PCR amplification using type-specific or consensus primers have been described for HPV detection [Meyer et al. 1995; Karlsen et al., 1996; Lorincz, 1996; Gravitt et al., 1998]. In the hybridization approach, the assays normally include only the more common HPV types. Several sets of degenerate consensus primers that are able to detect most genital HPV types have been described for PCR-based detection [Ting and Manos, 1990; Jacobs et al., 1997]. Following PCR amplification, restriction fragment length polymorphism (RFLP) analysis of the PCR-generated fragments is then used for typing [Bauser and Manos, 1993; Meyer et al., 1995]. To increase sensitivity and specificity, combinations of PCR and hybridization approaches have also been adopted [Jacobs et al., 1995; Meyer et al., 1995].

Grant sponsor: the Veterans General Hospital-Taipei; Grant number: VGH87-402.

Tian-Shiang Wang's present address is Section of Hematology, Department of Medicine, Veterans General Hospital-Taichung, Taichung, Taiwan

*Correspondence to: Dr. Kong-Bung Choo, Center for Molecular Medicine, Department of Medical Research and Education, Veterans General Hospital-Taipei, Shih-Pai, Taipei, 11217, Taiwan. E-mail: MACROBUTTON HtmlResAnchor kbchu@vghtpe.gov.tw

Accepted 19 May 1999

We have previously described a simplified version of the PCR-based RFLP procedure for HPV typing in which the number of analytical bands in the often complex RFLP patterns generated by digestion of multiple restriction enzymes is reduced (i.e., reductional RFLP, or rRFLP). This is achieved by the used of a biotin-labeled primer in the PCR which, on subsequent staining, reveals only the biotin-labeled restriction fragments [Wang et al., 1997]. In this report, a further development of the rRFLP approach is described using a fluorescence-labeled primer and an automatic sequencer for computer-assisted typing. The procedure thus does not impose constraints on the analysis of the more common HPV types as frequently done in other typing protocols.

MATERIALS AND METHODS

Clinical Samples

In this study, patients attending the gynecology colposcopy clinic of the Department of Obstetrics and Gynecology of the Veterans General Hospital-Taipei were included. Cervical scrapes were collected from individual patients in 2.5 ml of saline buffer and were subjected to HPV analysis within 24 hr. The same set of specimens was also subjected to PAP smear cytological examination. In addition, biopsies were taken for histological examination.

PCR Primers and Amplification

Oligonucleotide primers used in this study were synthesized commercially. Degenerate HPV consensus primers MY09 and MY11 were used for PCR [Ting and Manos, 1990]. The size of PCR fragments generated was about 450 bp. For the purpose of simplifying the RFLP patterns, the MY11 primers were 5′-labeled with the fluorescence dye FAM. In each PCR reaction, a pair of human β-globin primers P331 (5′-CATGTGGAGACAGAGAGAC-3′) and P332 (5′-CCTGAAGTTCTCAGGATCCA-3′) was included that generated a 303-bp DNA fragment. Details for PCR detection of the presence of HPV in clinical cervical scrape samples have been described previously [Wang et al., 1997].

HPV Typing by RFLP

For HPV-positive cases, typing was carried out as follows. Aliquots of 2 μl of PCR products were subjected to digestion with 10 units each of *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, and *Pst*I, respectively, at 37°C for 2 hr or longer. At the end of the digestion period, equal aliquots of each restriction reaction products were pooled. Fluorescence (ROX)-labeled size markers were added to each mixture and 2-μl aliquots of the mixture were then analyzed in a 4% denaturing polyacrylamide gel in an ABI 377 Automatic Sequencer. The DNA profiles obtained were analyzed and documented with the ABI Genescan software. For HPV typing, a computer program designated "HPV typing" was developed using the commercial Delphi computer programming software. A computerized databank of DNA fragments (Table I) carrying the fluorescence-labeled restriction fragments derived from the digestion of all known genital HPV types with the five restriction enzymes described above was also generated.

TABLE I. Fluorescence-Labeled Restriction Fragments of Anogenital HPVs Used in the rRFLP-Based HPV Typing Analysis[a]

HPV type	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6
HPV-16	452	210				
HPV-26	455	102				
HPV-61	455	220				
HPV-62	449	217	108			
HPV-53	449	217	209			
HPV-57	449	296	145			
HPV-52	449	423	266			
CP8304	452	121	111			
LVX100	452	220	158			
LVX82	452	383	373			
HPV59	452	426	396			
ISO39	455	243	215			
MM4	455	290	243			
HPV44	455	300	223			
HPV40	455	300	244			
HPV68	455	376	103			
HPV69	455	376	223			
MM9	458	432	218			
HPV58	449	238	207	104		
HPV56	449	283	207	145		
HPV30	449	294	217	207		
HPV33	449	323	237	207		
HPV66	449	370	294	207		
HPV42	449	370	344	237		
HPV6b	449	385	237	217		
HPV67	449	423	266	237		
MM8	452	345	145	109		
HPV54	452	373	237	217		
CP6108	452	373	325	155		
HPV51	452	379	365	241		
MM7	452	383	373	317		
HPV35	452	426	297	269		
HPV55	455	223	207	104		
HPV39	455	330	134	103		
HPV45	455	376	213	134		
HPV70	455	376	243	106		
HPV43	455	422	376	176		
HPV18	455	435	376	213		
HPV34	458	334	179	154		
HPV11	449	370	237	217	207	
HPV32	449	370	325	323	237	
HPV31	452	328	288	240	210	
CP8061	452	360	323	217	109	
LVX160	455	376	243	213	106	
HPV13	455	376	329	243	213	127
HPV64	458	432	379	370	334	154

[a]Band 1 represents nondigested DNA fragment. Bands 2–6 were generated from the mixing of products of independent digestions with *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, and *Pst*I. The number of bands obtained was a factor of the total number of restriction enzyme cleavage sites each HPV PCR fragment contained.

RESULTS AND DISCUSSION

To detect the presence of HPV in an exfoliated cervical sample, we used the degenerate primers MY09 and MY11 from the L1 gene of the HPV genome. These primers were known to amplify most genital HPV types [Ting and Manos, 1990]. The MY11 degenerate oligonucleotides were 5′-labeled with a fluorescence dye for

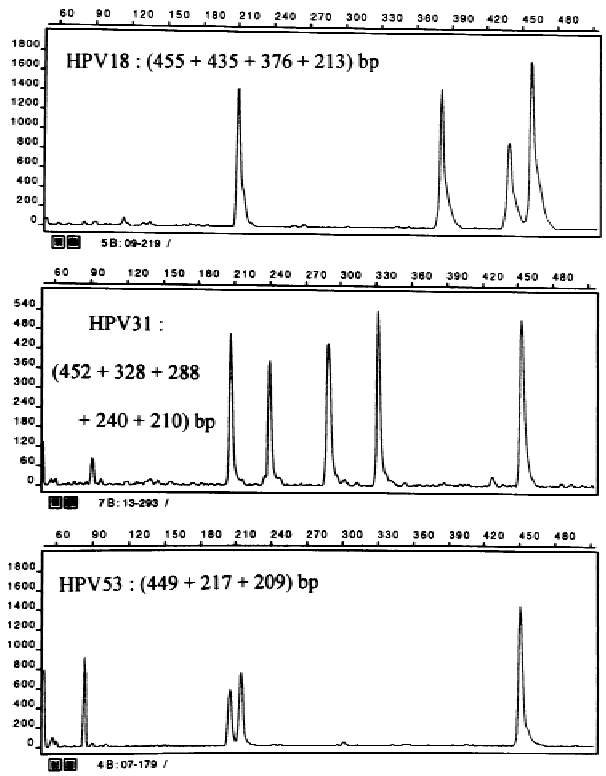

Fig. 1. Representative Genescan profile of HPV typing. Shown in the figure are three different HPV types with unique patterns of fluorescence-labeled restriction fragments. The fragment sizes are also shown. Fragments below 100 bp were excluded in the database analysis.

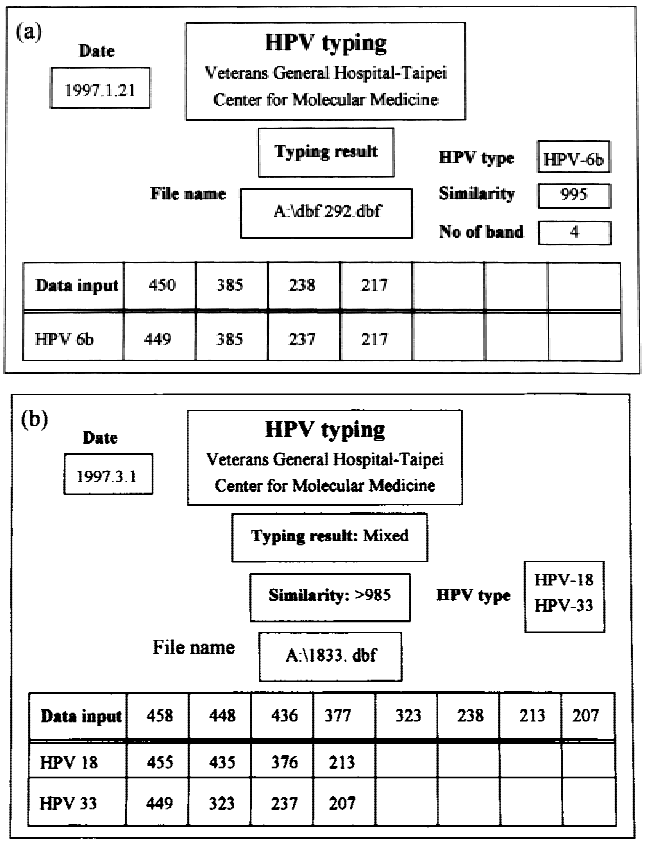

Fig. 2. Facsimiles (in English) of charts of computer-assisted sorting of HPV types in clinical samples. **a:** A case of single infection. **b:** A case of double infection. In the chart, the numericals in the data input row indicate the sizes of the fragments of the clinical sample being analyzed, whereas the row of numericals listed below are the closest match results of sorting from the databank of all HPV types. The HPV type of the sample is listed together with the degree of similarity between the best match and the sorted database pattern. The score for a perfect match has been set for 1,000.

subsequent detection and documentation. It is noteworthy that MY11 had been chosen over MY09 for labeling because longer fragments were more frequently produced at this end by restriction digestion. In the initial PCR detection step, a pair of unlabeled human β-globin gene primers was also included to ensure proper PCR amplification (data not shown; see Wang et al. [1997]).

To obtain a unique RFLP pattern for each HPV type, five restriction enzymes were selected [Wang et al., 1997]. The analysis of the RFLP patterns was further simplified when DNA fragments from the multiple restriction digestions were mixed and electrophoresed in a single lane in the automatic sequencer. For accurate sizing of the DNA fragments, size markers labeled with a different fluorescence dye were mixed with each sample and electrophoresed in the same lane. In an automatic sequencer, the multiple labeled DNA fragments were well resolved and were accurately sized (Fig. 1). Each HPV type was associated with a unique rRFLP pattern (Fig. 1 and Table I). In our analysis, the original undigested PCR products (measuring between 449 and 458 bp) were always included as a baseline reference (band 1 in Table I). To reduce the noise level in the scan run, restriction fragments below 100 bp were discarded without affecting the uniqueness of the rRFLP patterns for the HPV types included (see Table I). Thus, each HPV type has an identification tag consisting of two to six different restriction bands, including the undigested band 1. The presence of the control β-globin gene fragments, whether or not digested by any of the five restriction enzymes, did not show up in the final fluorescence rRFLP patterns. The accuracy of the rRFLP approach for HPV typing was confirmed by sequencing of amplified fragments of 30 randomly chosen cases. Complete agreement was obtained (data not shown).

For computer-assisted typing of clinical samples, a database of fluorescence-labeled rRFLP patterns of 46 known anogenital HPVs was constructed (see Table I). A simple matching program was developed using the commercial Delphi software. The Genescan profile of each sample was analyzed with the HPV Typing Program against the database for matches. A parameter designated "extent of similarity," with 1,000 for a perfect match, in the sample and database matching was included in the HPV Typing Program for manual evaluation of confidence of the accuracy of each match. In most cases with infection of a single HPV type, a single HPV identification was made, together with a

TABLE II. Distribution of Different HPV Types

HPV type	Number	Frequency (%)
Low risk (n = 21; frequency = 15.1%):		
6b	10	7.19
CP8304	4	2.88
MM7	2	1.44
61	2	1.44
62	1	0.72
11	1	0.72
CP8061	1	0.72
High risk (n = 115; frequency = 82.8%):		
16	46	33.09
33	15	10.79
58	15	10.79
18	11	7.91
31	6	4.31
52	5	3.60
53	5	3.60
39	3	2.16
70	3	2.16
66	2	1.44
68	2	1.44
MM4	1	0.72
56	1	0.72
Other (n = 3; frequency = 2.1%):		
Untyped	3	2.16
Total	139	100

computed extent of similarity and the number of bands used in the match (Fig. 2a). In such single HPV type matching, a similarity of 990–1,000 (99%–100% similarity) was normally achieved and accepted. In the rare cases of mixed HPV types, the program automatically switched to a subprogram for sorting of band identity from more than a single HPV type in the database (Fig. 2b). In such cases, a slightly lower similarity (ca. 98.5%) than in the single-type cases was normally observed and accepted. The sizing was highly accurate as demonstrated by resolution of the 209- and 217-bp peaks in the HPV-53 panel in Figure 1. There were also a few occasional cases in which the matching failed to identify a known HPV type from the database. These cases were then marked "untyped." The untyped cases were subsequently shown by sequencing to represent novel or variants of known HPV types (data not shown).

We have applied the established procedure and database to the typing of 133 HPV-positive clinically obtained PAP smear cases. A total of 20 different HPV types were detected (Table II). Of these, 15.1% belonged to the low-risk group, whereas the majority of the HPV types detected belonged to the intermediate- to high-risk group. There were six cases of double HPV-type infection and three untyped cases. In the low-risk group, HPV-6b and CP8304 were more prevalent,

TABLE III. Prevalence of HPV Types in Relation to Different Pathological Grades of Cervical Lesions

	Number of cases						
HPV type	Normal	Flat Condyl	CIN I	CIN II	CIN III	Ca	Total
Low-risk HPV types (n = 20; frequency = 15.0%):							
6b	8	1	1	0	0	0	10
CP8304	2	0	1	0	0	1	4
MM7	2	0	0	0	0	0	2
61	2	0	0	0	0	0	2
62	1	0	0	0	0	0	1
11	0	1	0	0	0	0	1
High-risk HPV types (n = 104; frequency = 78.2%):							
16	6	2	0	3	20	13	44
58	1	1	0	1	7	5	15
18	2	1	1	1	4	1	10
33	4	0	0	3	4	1	12
31	0	0	0	0	4	2	6
52	1	1	0	1	1	1	5
53	1	1	0	1	1	0	4
70	2	0	0	0	1	0	3
39	0	0	1	0	0	0	1
56	0	0	1	0	0	0	1
66	0	0	0	1	0	0	1
68	1	0	0	0	0	0	1
MM4	0	0	0	0	1	0	1
Mixed HPV types (n = 6; frequency = 4.5%):							
CP8061 + 68	0	1	0	0	0	0	1
16 + 33	0	0	0	0	1	0	1
16 + 39	0	0	0	0	1	1	2
18 + 33	0	0	0	0	1	0	1
33 + 53	0	0	0	0	1	0	1
Untyped (n = 3; frequency = 2.3%):							
Untyped	2	0	0	0	1	0	3
Total	35	9	5	11	48	25	133

whereas in the high-risk group, HPV-16 constituted a third of all HPV cases detected. HPV-18, -33, and –58 were also prevalent in the high-risk group. In some of the commercial HPV typing kits currently available, HPV-6b, -16, -18, -33, and –58 are included as some of the more common HPV types. However, it is evident from our analysis that there is still a significant number (about one-fifth of all cases) of other, less common HPV types not normally included in commercial kits.

When the HPV types detected were further analyzed against the background of the pathology of the cervical lesions (Table III), it is observed that most low-risk HPV cases were associated with cytologically normal or low-grade cervical lesions with only one case of exception. A single case of infection by the low-risk CP8304 was found to be associated with a cervical cancer case. CP8304 was originally cloned from a woman with a normal cervical diagnosis in New Mexico [Peyton and Wheeler, 1994]. The CP8304 strain found in our cancer case may be an oncogenic variant of this normally low-risk HPV type. An alternative explanation is that the original New Mexico strain could be an attenuated variant of the otherwise high-risk HPV type.

As anticipated, the majority of the high-risk HPV infection cases were found in grade III cervical intraepithelial neoplasia (CINIII) or cancer. HPV-16 showed the highest prevalence rate, followed by HPV-18 and –33, consistent with previous reports on cases in Taiwan [Chen et al., 1994; Liaw et al., 1995]. Our study also reveals a significant prevalence of HPV-31, -52, -53, and -58, as has been described [Huang et al., 1997]. The few cases of infection of high-risk HPV types in cytologically normal cases are high-risk cases for developing higher-grade lesions and even cancers [Hildesheim et al., 1994; Remmink et al., 1995] and are now being closely monitored. In the six cases of double infection, the most common HPV types such as HPV-16 and -18 were involved. It thus comes with no surprise that these double infection cases were associated with CINIII and cancer.

In summary, this report describes a development in the PCR-based RFLP approach for HPV typing using fluorescence-labeled primers and automated matching of the HPV type through electrophoretic analysis in an automatic sequencer. There are no constraints on the analysis of only the more common HPV types in the procedure. For a more comprehensive identification of HPV infection, HPV detection and typing methods such as that described here need to be developed and employed for routine clinical HPV typing.

ACKNOWLEDGMENT

We thank Ms. San-Huai Chiang for her involvement in the development of the computer program.

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